Chapter 5

Summary and concluding remarks

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"γ-Butyrolactones and their role in regulation"
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The search for new drugs against pathogenic bacteria causing infectious diseases will probably never end. At best, we are always one step ahead in the race between resistant pathogens and novel antibiotics. In recent years, reports of "superbugs", deadly pathogens against which no known drug is effective any longer, alarmed both the scientific community as well as the general public. For various reasons the number of genuinely new antibiotics on the market has been declining for decades (Fig. 1 in chapter 1), while development of resistance among pathogenic bacteria increased. Therefore, the search for pharmaceutically active natural products has moved back into the centre of scientific interest (Hopwood, 2007).

More than three quarters of the clinically used antibiotics are natural products or slightly modified natural products (Newman *et al.*, 2003). These drugs originally derive mostly from fungi and a group of soil bacteria, the streptomycetes, which produce antibiotics as secondary metabolites (Fig. 1 in chapter 1) (Hopwood, 2007). However, since their biosynthesis is complex and "costly" for the producing organism, secondary metabolite production is strictly regulated in the microbial cell.

The aim of this thesis was (1) to gain further insight into the *Streptomyces coelicolor* butanolide system, a central antibiotic regulatory system in the model organism of its genus (reviewed in Takano, 2006), and (2) to trigger production of novel antibiotics by manipulation of the regulation of their biosynthetic gene clusters.

The role of ScbR in a bacterial hormone system of *Streptomyces coelicolor*

Secondary metabolite production in *Streptomyces* is controlled by a complex regulatory network. One of the regulatory systems of antibiotic production is based on "bacterial hormones", the γ-butyrolactones. The small signalling molecules are produced in and spread among the cells of a *Streptomyces* colony. This may ensure that all cells start simultaneously with the production of antibiotics and therefore increase its efficiency (Takano, 2006). Furthermore, mechanisms of self-resistance may be activated prior to the production of antibiotics (Y. Ohnishi, personal communication).

In the model streptomycete *S. coelicolor* this regulatory system is formed by three main components: first, a "sender", the ScbA protein, involved in the production of the signalling molecules. Secondly, the bacterial hormones themselves; and, thirdly, a "receiver" or receptor, the ScbR protein, the *Streptomyces coelicolor* butanolide receptor (Fig. 1; Fig. 4 and 5 in chapter 1) (Takano, 2006).

Fig. 1 illustrates the mode of action of ScbR: A ScbR homodimer acts as negative transcriptional regulator by binding to specific sites upstream of its target genes via a DNA binding domain (Fig. 1a). In the presence of the γ-butyrolactones, the signalling molecules bind to the ligand binding pocket of ScbR, altering the structural conformation of ScbR and in particular the DNA binding domain so that the receptor can no longer bind to DNA (Horinouchi, 2007). Consequently, the bacterial hormones have caused a de-repression of the ScbR target genes and the initially silenced genes are now expressed (Fig. 1b) (Takano *et al.*, 2001; Takano, 2006).

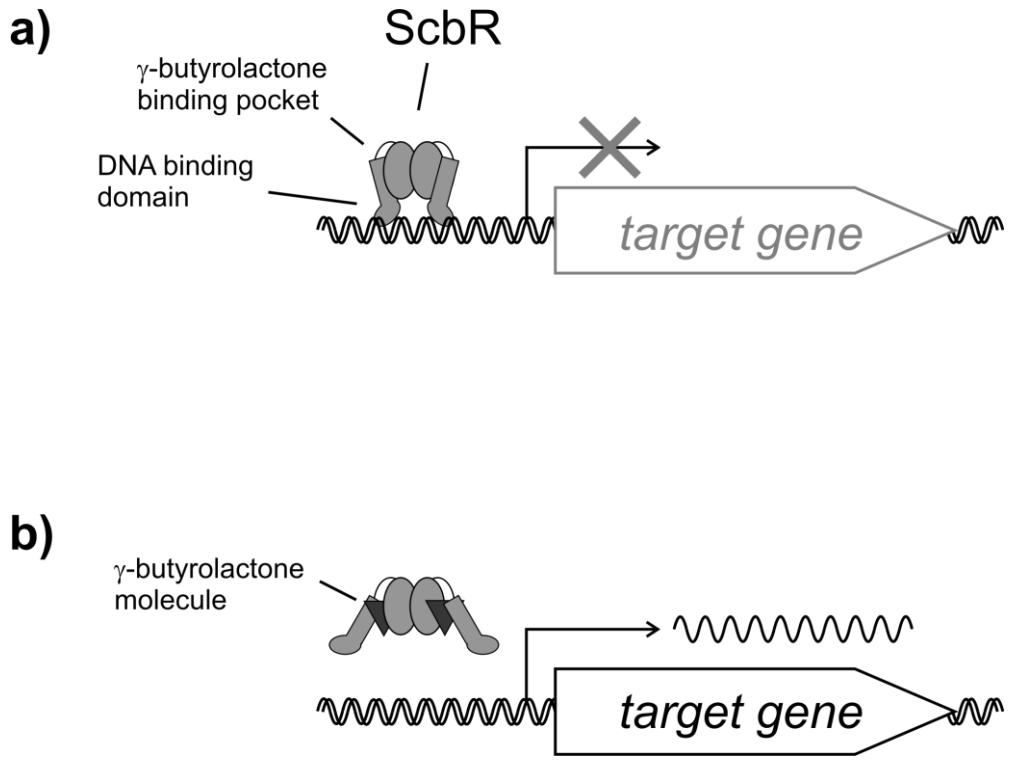

Figure 1 Mode of action of the γ-butyrolactone receptor and transcriptional regulator ScbR in *Streptomyces coelicolor* (explanation in the text)

ScbR, *scbA* and *cpkO* have been experimentally identified as target genes of ScbR (Fig. 5 in chapter 1) (Takano *et al.*, 2001; Takano *et al.*, 2005a). ScbA most likely catalyses the condensation of dihydroxyacetone phosphate (DHAP) and a β-keto acid derivate which comprises the first step in the biosynthesis of the γ-butyrolactones (Fig. 2) (Hsiao *et al.*, 2007). CpkO is the pathway specific regulator of the *cpk* secondary metabolite gene cluster (Fig. 2 in chapter 1) (Pawlik *et al.*, 2007). Thus, in addition to its own expression, ScbR also regulates the production of the signalling molecules of the *S. coelicolor* butanolide system, as well as a putative antibiotic biosynthesis gene cluster. ScbR was also shown to affect production of two pigmented *S. coelicolor* antibiotics, the blue-coloured actinorhodin (Act) (Bystrykh *et al.*, 1996; Rudd and Hopwood, 1979), and the red-pigmented prodigiosins (Red) (Feitelson *et al.*, 1985) (Fig. 4 in chapter 1).

In total four binding sites were identified in front of the three ScbR target genes: Site R in front of *scbR*, site A in front of *scbA* and site O_A and site O_B in front of *cpkO* (Takano, 2006). Site R and site O_A show an identical inverted repeat sequence,

whereas site O_B and even more site A are less congruent with this ScbR binding sequence. *In silico* analysis predicted a single additional fully conserved ScbR binding site in the *S. coelicolor* chromosome in front of *orfB* (Takano *et al.*, 2005a). *OrfB* is located in close vicinity of *scbR*, separated only by two genes, *scbA* and *scbB*. Binding of ScbR in front of *orfB* was confirmed in gel retardation assays (Takano, personal communication). The function of the predicted histidine kinase OrfB, however, is not known. Recent preliminary results indicate that OrfB is involved in a ScbR deactivation mechanism by modification (possibly phosphorylation) of ScbR (Takano, personal communication). Interestingly, only for the conserved binding sites, site R and site O_A, ScbR has been confirmed as transcriptional repressor. ScbR binding at site A was suggested to possibly promote activation (Takano *et al.*, 2001), and preliminary *in vitro* data indicate a corresponding function of ScbR also at site O_B (Takano, personal communication). However, the putative dualistic function of ScbR at each two binding sites (repressor at site R and site O_A, activator at site A and site O_B) is not consistent with the position of the sites. Site A and site O_A overlap with the -10 and -35 region of the transcriptional start site of *scbA* and *cpkO*, respectively, whereas site R and site O_B are located -42 nt to -68 nt and -222 nt to -244 nt upstream of *scbR* and *cpkO*, respectively.

$ScbR_{M600}$ - Discovery and investigation of a second form of the *S. coelicolor* γ-butyrolactone receptor

After decades of *S. coelicolor* research, a whole family tree of strains of the species is available, all deriving from the original isolate (Waksman strain W3443) (Weaver *et al.*, 2004). *S. coelicolor* M145 is the typical laboratory strain that was fully sequenced (Bentley *et al.*, 2002), but also strain M600 is often used in many research groups.

When analysing the proteome of these two *S. coelicolor* strains by 2D gel electrophoresis, we identified in M600 a variant of ScbR ($ScbR_{M600}$) that is mutated in the vicinity of the γ-butyrolactone binding pocket compared to ScbR from M145 ($ScbR_{M145}$). A single amino acid change, Arg120Ser, in $ScbR_{M600}$ was revealed in MALDI-TOF analysis. A corresponding point mutation was confirmed by DNA sequence analysis in $scbR_{M600}$ of independent isolates of strain M600 provided by

several laboratories. The same mutation has also been found in the *scbR* gene of strain A3(2) N2.

Possible effects of the mutation in $ScbR_{M600}$ were examined *in vitro* and *in vivo*. For *in vitro* characterisation, we cloned and expressed both *scbR* variants in *E. coli* and used crude cell extracts to compare the properties of $ScbR_{M145}$ and $ScbR_{M600}$ in gel retardation assays. Contrary to our expectations, the mutation resulted in an instable DNA binding activity of the transcriptional regulator. This occurs despite the mutation's location near the ligand binding pocket and not in the vicinity of the DNA binding domain. Surprisingly, binding of the γ-butyrolactones, on the other hand, was not affected in $ScbR_{M600}$ (chapter 4).

From the protein structure of the ScbR homologue CprB (Horinouchi, 2007; Natsume *et al.*, 2004), amino acid 120 in ScbR (Glu126 in CprB (Fig. 6 in chapter 1)) was deduced to be part of α-helix 7 in the regulatory region of the protein (Fig. 2 in chapter 4). The residue faces the protein surface and is distant from the DNA binding and the dimerisation domain; it's only functional feature seems to be that it is the neighbouring residue to Trp121 in ScbR (Trp127 in CprB (Fig. 6 in chapter 1), a residue crucial for ligand binding (Sugiyama *et al.*, 1998) (D. Linke, personal communication, based on (Natsume *et al.*, 2004)). Therefore, the nature of the structural effect of the mutation in $ScbR_{M600}$ leading to the quick loss of DNA binding *in vitro* (chapter 4), remains unclear. Protein crystallization of the γ-butyrolactone receptor homologues is difficult and consequently the structure of CprB remains the only one available. Co-crystallization of both variants of ScbR with its cognate ligand and also with its DNA target region would lead to a better understanding of the structure-function relationship of these proteins and is therefore of great interest in future work.

For *in vivo* characterisation, and to avoid the influence of a different chromosomal background and the effects of putative further mutations in the genome of *S. coelicolor* M600, the mutated *scbR* locus in a M145Δ$scbR_{M145}$ in-frame deletion mutant (M752) (Takano *et al.*, 2001) was replaced by $scbR_{M600}$ and $scbR_{M145}$ yielding strains LW33 and LW34, respectively. LW33 therefore encodes $ScbR_{M600}$ in the M145 genetic background; LW34 is the control strain expressing the native $ScbR_{M145}$. *In vivo*, in agreement with our *in vitro* results, DNA binding activity of

$ScbR_{M600}$ was impaired. Quantitative RT-PCR analysis showed delayed transcription of the pathway-specific regulatory genes of the *act*, *red* and the *cpk* biosynthesis gene clusters in strain LW33. However, production levels of the related secondary metabolites did not differ significantly between strains LW33 and LW34. Also, expression of *scbA* involved in the synthesis of the γ-butyrolactones was delayed in LW33, and consistently a γ-butyrolactone bioassay indicated a minor decrease in the production of the signalling molecules (chapter 4).

Regulation of the *act*, *red* and *cpk* gene clusters by the butanolide system is intertwined with the complex regulatory network of secondary metabolism in *S. coelicolor*. The *cpk* gene cluster, for example, was shown to be also regulated by the RapA1/A2 two-component system (Lu *et al.*, 2007) and the global regulator DasR (Rigali *et al.*, 2008). PhoP, the response regulator of the PhoR/P two-component system related to phosphate metabolism, binds to an intragenic DNA region of a *cpk* biosynthesis gene and is required for the transcription of the *cpk* genes (Colin Smith, personal communication). Furthermore, production of other, known and unknown, secondary metabolites in *S. coelicolor* may impair the synthesis of Act, Red and the *cpk* end product, e.g. by competition for common precursors from primary metabolism. Precursor competition would affect production of Act, Red and the *cpk* product even when the related biosynthesis genes are highly expressed, thereby overriding the transcriptional regulation of the *act*, *red* and *cpk* gene clusters. All this may explain why the comparably small changes in gene expression of their pathway-specific regulatory genes caused by the mutation in $ScbR_{M600}$ did not result in an apparent change in the antibiotic phenotype (chapter 4).

$ScbR_{M600}$ was initially identified in 2D gel analysis in the proteome of spores of *S. coelicolor* M600. Surprisingly, $ScbR_{M600}$ was never detected in mycelial samples of M600, whereas in M145 $ScbR_{M145}$ is present in the mycelium (chapter 4), but was never found in spores (A. Hesketh, personal communication). This striking difference is most probably not caused by the mutation in $ScbR_{M600}$, but is due to the M600 genetic background, since $ScbR_{M600}$ was present in the mycelium when expressed in a M145 genetic background in LW33 (chapter 4). Intracellular conditions in the dormant spores may differ from those in growing mycelial cells. Although studied in

vegetative mycelium, the $ScbR_{M600}$ specific protein characteristics may therefore represent an adaptation of the protein to the spores where expression of $ScbR_{M600}$ was detected and thus to its natural cellular environment. *In vivo* activity of $ScbR_{M600}$ in spores has not been shown yet, and one could further speculate that the role of $ScbR_{M600}$ in the spores, where no antibiotics are produced, differs from that described for $ScbR_{M145}$ in the mycelium. A M600Δ*scbR*$_{M600}$ mutant, as well as the expression of *scbR*$_{M145}$ in a M600Δ*scbR*$_{M600}$ genetic background would be useful to address these hypotheses in future work. Putative effects on development, especially on sporulation and germination, but also changed properties of the spores (e.g. heat resistance) would be of special interest.

In the lineage of the *S. coelicolor* laboratory strains, two major changes in the genome are most apparent: the loss of the two naturally occurring *S. coelicolor* plasmids, the linear SCP1 and the circular SCP2, and the duplication of a 1.06 Mb region from the left chromosome end. The duplication contains 1005 genes, among them four gene clusters known or predicted to be related to secondary metabolites (eicosapentaenoic acid, isorenieratene, deoxysugar synthases/glycosyl transferases, and coelichelin) (Bentley *et al.*, 2002). Transcript levels of the duplicated genes, however, are similar in strains with or without the duplication, and few obvious phenotypic differences exist (Weaver *et al.*, 2004). On the *S. coelicolor* plasmids, we find secondary metabolite genes, i.e. the methylenomycin (Mm) biosynthesis gene cluster on SCP1 (Bentley *et al.*, 2004). Mm production is controlled by a recently discovered regulatory system encoded in the Mm gene cluster that is similar to the *S. coelicolor* butanolide system. This system is based on a novel class of antibiotic biosynthesis inducers, the 2-alkyl-4-hydroxymethylfuran-3-carboxylic acids (AHFCAs), collectively called Mm furans (MMFs), with analogous functions to the γ-butyrolactone regulatory molecules. Also ScbR-like receptor and transcriptional regulator proteins (MmyR and MmfR) were identified (Corre *et al.*, 2008). Given the encoded functions, the chromosomal duplication and the presence or absence of SCP1 plasmid may influence antibiotic production and regulation. Therefore one could speculate that the mutation in $ScbR_{M600}$ is related to these genomic alterations. *S. coelicolor* M145 and M600 lost the plasmids; strain A3(2) N2 possesses SCP1 and SCP2. Interestingly, strains M600 and A3(2) N2 (*scbR*$_{M600}$) possess the duplication, whereas M145 (*scbR*$_{M145}$) is the only one without the prolonged

chromosome. A putative correlation between the duplication and the mutant *scbR*$_{M600}$, however, could not be confirmed, since in 7 additional *S. coelicolor* strains with the duplication (Weaver *et al.*, 2004), *scbR*$_{M145}$ was identified (chapter 4).

Although unknown, a natural relevance for the amino acid change Arg120Ser in ScbR of strain M600 and A3(2) N2 seems likely, since this was the only variant form of the protein found and based on the lineage of *S. coelicolor* (Weaver *et al.*, 2004) the mutation occurred independently in the two strains. Also, strains M600 and A3(2) did not undergo any mutagenic treatment (Weaver *et al.*, 2004).

The *cpk* gene cluster as the actual target of the *Streptomyces coelicolor* butanolide system

Usually, production of the two coloured antibiotics Act and Red is investigated in *S. coelicolor*. There is only one additional antibiotic known in *S. coelicolor* M145, the colourless calcium-dependent antibiotic, CDA (Hopwood and Wright, 1983; Lakey *et al.*, 1983). Although Act and Red production is influenced by the *S. coelicolor* butanolide system, it becomes more and more evident that none of these antibiotics is the actual target of the regulatory system (Takano, 2006).

Genome analysis of *S. coelicolor* M145 has identified many other secondary metabolite gene clusters in addition to those required for the production of Act, Red and CDA (Bentley *et al.*, 2002). Most of them, however, are still “orphan” (Gross, 2007) and the metabolites produced by the encoded biosynthetic pathways are unknown. Most likely, these gene clusters are not expressed under typical growth conditions. Alternatively, the biosynthesis genes may well be expressed, but the related compound has not been identified. One of these orphan gene clusters is the *cpk* gene cluster. CPK denotes for *coelicolor* polyketide. Polyketides compose a group of chemical compounds to which numerous antibiotics are assigned and it can also be assumed that the *cpk* gene cluster encodes for a polyketide. However, no details about the chemical structure of the substance can be predicted, nor was it known when this secondary metabolite is produced by the bacterium and whether the natural compound shows antibiotic activity (Pawlik *et al.*, 2007).

There is evidence that the butanolide system in *S. coelicolor* primarily provides regulation for the *cpk* cluster. The previously observed effects on the production of Act and Red seem to be downstream effects only, e.g. by competition for common precursors (chapters 2 and 3, and (Takano, 2006). The γ-butyrolactone receptor ScbR binds upstream of *cpkO*, the pathway-specific activator of the *cpk* cluster (Takano *et al.*, 2005a) and represses its expression. For Act and Red, on the other hand, such direct regulation could not be shown (Takano *et al.*, 2001). Interestingly, *scbR2* was identified in the *cpk* gene cluster (Fig. 2 in chapter 1). ScbR2 shows 32% amino acid identity with $ScbR_{M145}$. Based on amino acid sequence similarity, ScbR2, together with e.g. TylQ from *S. fradiae* and BarB from *S. virginiae*, appear to belong to a subfamily of γ-butyrolactone receptor homologues that act as transcriptional regulators in the absence of ligand binding (Takano *et al.*, 2001).

The presence of several γ-butyrolactone receptor homologues in a *Streptomyces* strain or even in a single gene cluster is not unusual. The tylosin biosynthetic gene cluster in *S. fradiae* contains two, TylP and TylQ (Bignell *et al.*, 2007), of which TylQ is proposed to be a pseudo-receptor without ligand binding ability. AlpW and the pseudo-receptor AlpZ in the alpomycin gene cluster in *S. ambofaciens* (Bunet *et al.*, 2008) are another example. In addition to ScbR and ScbR2, which are both involved in the regulation of the *cpk* gene cluster (Takano *et al.*, 2005a; chapter 2), *S. coelicolor* possesses at least 3 other chromosomally encoded ScbR homologues (CprA, CprB, SCO6323). CprA and CprB have been reported to affect Act and Red production and sporulation in *S. coelicolor* (Onaka *et al.*, 1998) and based on their typical alkaline pI values (pI 9.53 and 9.77, respectively) are supposed to be pseudo-receptors (Kitani *et al.*, 2008). CprA and CprB have been shown *in vitro* to bind to site O_A in front of *cpkO* in *S. coelicolor* and thus share at least one target site with ScbR (Takano, personal communication). Furthermore, CprA and CprB bind the DNA target of ArpA from *S. griseus* (Sugiyama *et al.*, 1998). Horinouchi and co-workers even suggested that most of the receptor homologues recognize and bind the same target DNAs because of the great similarity of the amino acid sequence consisting of the helix–turn–helix DNA recognition domain (Nishida *et al.*, 2007). Assuming corresponding functions for CprA, CprB and putatively SCO6323 also *in vivo* would add a whole new level of complexity to the regulatory processes taking place at the ScbR target sites in *S. coelicolor*.

yCPK - Triggering the production of a novel natural product in *Streptomyces coelicolor*

Given the important role of ScbR for antibiotic production in *S. coelicolor* we investigated the function of ScbR2. A *scbR2* deletion mutant was created and conditionally produced a yellow pigment which was not observed previously, replacing the red (Red) and blue (Act) antibiotics. To confirm that production of the yellow compound resulted solely from the deletion of *scbR2*, *scbR2* was reintroduced into M145Δ*scbR*. Act, Red and yellow compound production was restored to the levels observed in the parental strain M145. Under specific growth conditions, the yellow pigment was also produced in M145, however, in much lower levels. Using a newly created *act*, *red*, *cda* triple antibiotic biosynthesis gene cluster deletion mutant, the yellow substance was shown not to be related to the three characterized *S. coelicolor* secondary metabolites. Additional deletion of single *cpk* genes or of the entire *cpk* cluster, however, resulted in disappearance of the yellow colour (Fig. 4 in chapter 3). Therefore, we concluded that production of the yellow pigment (yCPK (yellow *c*oelicolor polyketide)) requires a functional *cpk* gene cluster and thus assigned yCPK to the hitherto orphan gene cluster.

yCPK production in the parental M145 was observed for only a few hours during an early stage of growth, whereas in the *scbR2* mutant production was clearly prolonged (Fig. 4 in chapter 3). This may be the reason why, despite comprehensive metabolite analysis of *S. coelicolor*, the yellow pigment has not been described earlier and the *cpk* gene cluster remained orphan. In agreement with the observed yCPK production, transcription studies using quantitative RT-PCR analysis indicated that ScbR2 represses expression of *cpkO* and consequently also of biosynthetic genes in the *cpk* cluster (Fig. 6 in chapter 3). Together with the activator CpkO, ScbR2 constitutes “negative feedback regulation” of yCPK production: CpkO activates the *cpk* genes that will provide for the production of the yellow pigment. Slightly delayed, CpkO also activates *scbR2*. ScbR2 eventually represses *cpkO* and thus the production of yCPK and its own expression. And since *cpkO* is regulated by ScbR this control cycle is directly interlinked with the *S. coelicolor* butanolide system (Fig. 2; chapter 3).

abCPK - A new antibiotic from *Streptomyces coelicolor*

After the discovery of the novel yellow pigment, growth conditions were optimized for the production of yCPK. In particular, supplementing the culture media with glutamate increased yCPK production significantly (Fig. 2 in chapter 3). On the contrary, in medium with casamino acids yCPK production was suppressed (Fig.1 in chapter 3). This clearly suggests an effect of the nitrogen source on the *cpk* biosynthetic pathway (chapter 3). A possible explanation for the stimulating effect of glutamate is discussed later in this chapter. Different nitrogen sources including other amino acids should be used to characterise this dependency and possibly further increase the production of the *cpk* metabolite. Feeding of radio-labelled glutamate might serve to demonstrate incorporation of the provided nitrogen in yCPK.

Coinciding with yCPK production under the optimised conditions we also observed a previously undetected antibacterial activity against Gram+ and Gram- bacteria in *S. coelicolor*. Bioactivity tests using, for example, the Δ*act red* (*cpk*) *cda* mutants revealed that also the antibiotic activity is related to the *cpk* gene cluster (Fig. 6 in chapter 3). Furthermore, increased antibiotic production was observed with the yCPK overproducing *scbR2* mutant (Fig. 7 in chapter 3). Thus, at first, it seemed that yCPK is not only a novel pigment, but actually a yet undiscovered antibiotic in *S. coelicolor*.

The yellow substance was partially purified to further investigate its properties. Surprisingly, the yellow pigment yCPK, as well as an antibacterial substance (abCPK (antibiotic *coelicolor* polyketide)) were isolated separately, and thus these are not identical metabolites. In HPLC analysis the yellow and the antibacterial *cpk* compounds were detected in different fractions, and yCPK could never be shown to have antibiotic activity (data not shown). Furthermore, yCPK is only found secreted into the medium in *S. coelicolor* liquid cultures (Fig. 5 in chapter 3), whereas active abCPK can be isolated from cells lacking the yellow pigmentation with polar solvents such as methanol (Fig. 8 in chapter 3). However, we explicitly do not exclude the possibility that abCPK is present outside, or attached to, the cell. Even more importantly, yCPK and abCPK production was uncoupled by deletion of the *cpk* biosynthesis gene *scF*. In the *scF* mutant, yCPK production is abolished whereas

abCPK activity was still observed. In contrast, a mutant in the *scF* homologue *cpkH* produced neither the yellow pigment nor the antibiotic (Fig. 7 in chapter 3). ScF and CpkH are predicted secreted oxidoreductases (Pawlik *et al.*, 2007) and are substrates of the *S. coelicolor* Tat protein secretion system (Widdick *et al.*, 2006). We therefore propose that the last steps of the biosynthesis of the *cpk* products take place outside of the cell. There, CpkH catalyses the formation of abCPK that is subsequently converted to yCPK by ScF. We have stopped yCPK synthesis and simultaneously increased the production of abCPK (chapter 3) which may facilitate high-yield extraction, purification and structure elucidation of the antibacterial *cpk* metabolite in future work. Subsequently, the potential of the novel antibiotic agent as a pharmaceutical product will be further characterised.

Our results suggest that the antibiotic compound (abCPK) is an intermediate and not the end product of the *cpk* biosynthetic pathway (Fig. 2; chapter 3). This raises questions about the natural function of abCPK. Due to the immediate conversion of the antibiotic into a yellow pigment, for which no antibacterial activity could be shown, a specific role as antibacterial agent secreted by *S. coelicolor* seems unlikely. It may well be that the intermediate abCPK is nothing more than a “shunt product” of the *cpk* biosynthetic pathway. Therefore, the unknown role of the putative *cpk* end product yCPK is of high interest and remains to be further investigated. As mentioned above, results presented in chapters 2 and 3 suggest that yCPK does not possess antibacterial activity. It is produced by M145 grown in darkness and only during a short period of growth. Therefore also a role in the protection of cells from photodamage, similar to the function of yellow carotenoids in *S. coelicolor* (Takano *et al.*, 2005b), appears unlikely. It has been suggested that the majority of low-molecular-weight organic compounds made and secreted by microbes, and even many of those that show antibiotic activity, play roles as cell-signalling molecules in the environment and regulate gene expression in microbial populations, and possibly the interactions of these populations with the surrounding organisms (Yim *et al.*, 2007). This idea is particularly fascinating when conferred on the *cpk* products which themselves are controlled by the γ-butyrolactone signalling molecules. However, no experimental data are available and thus one can only speculate about the function of yCPK as well as about a putative additional or alternative role for the antibacterial abCPK.

A hypothetical model of the *cpk* biosynthesis pathway and its regulation

Figure 2 shows a comprehensive hypothetical model of the *cpk* biosynthetic pathway and its regulation. The model summarizes the results presented in this thesis (chapters 2 and 3) and in relevant publications, amino acid sequence (homology) analysis of the *cpk* gene products (using blastp (http://blast.ncbi.nlm.nih.gov/Blast.cgi (Ye *et al.*, 2006), http://www.expasy.org/tools/blast/ (Gasteiger *et al.*, 2003)), Tatfind (http://signalfind.org/tatfind.html (Rose *et al.*, 2002)), SignalP (http://www.cbs.dtu.dk/services/SignalP/ (Bendtsen *et al.*, 2004)) (data not shown) and (Pawlik *et al.*, 2007)) and computational prediction of the functions of Cpk proteins in cellular processes of *S. coelicolor* (using KEGG (Kanehisa *et al.*, 2009) and BRENDA (Chang *et al.*, 2009)).

CpkPα and CpkPβ are predicted to form together the branched-chain α-keto acid dehydrogenase (BCDH) complex. The CpkP$\alpha\beta$ holoenzyme is mapped to catalyse the conversion of 2-oxoglutarate into succinyl-CoA in the TCA cycle of *S. coelicolor* (KEGG). Interestingly, homologues of the putative class-III aminotransferase CpkG were shown to form 2-oxoglutarate from glutamate (BLAST, ExPASy, BRENDA). Thus, CpkG may catalyse a direct anaplerotic reaction of the TCA cycle. However, both reactions are reversible and thus, CpkP$\alpha\beta$ and CpkG could also provide glutamate from TCA cycle intermediates. Glutamate and other amino acids could also be used by CpkG as donor substrate in a direct transamination (Pawlik *et al.*, 2007;Yonaha *et al.*, 1992) of a metabolite of the *cpk* biosynthesis pathway. A corresponding hypothetical role for CpkG is discussed in detail later and is denoted in Fig. 2. Any of these proposed enzymatic functions may be involved in the stimulating effect of glutamate on the production of the *cpk* products reported in chapters 2 and 3. Clearly, more work is needed to elucidate the dependency of yCPK and abCPK production on primary metabolism and on specific nitrogen sources in particular.

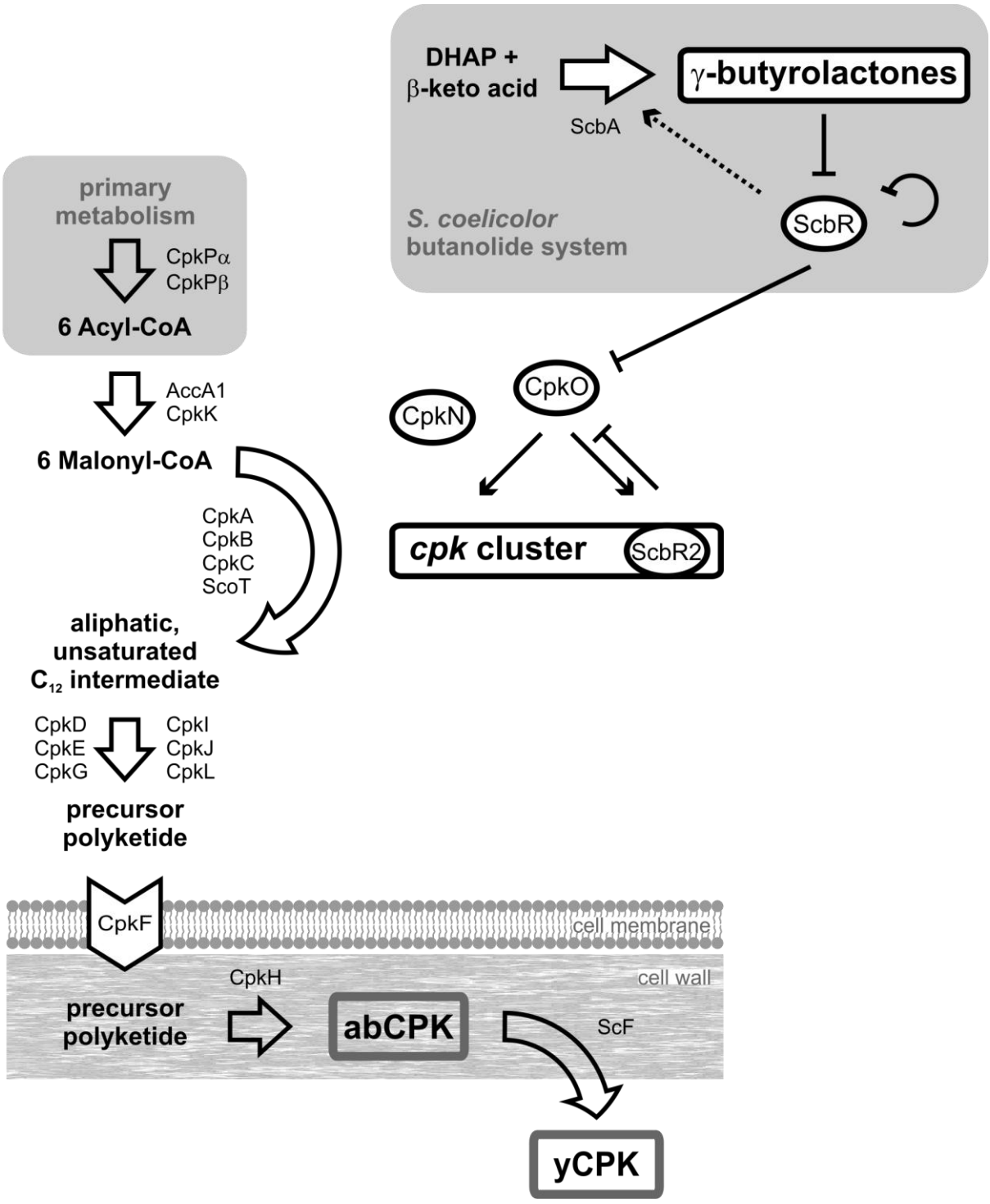

Figure 2 Hypothetical model of the cpk biosynthetic pathway and its regulation (explanation in the text)

During antibiotic production the acetyl-CoA flux may be redirected from entering the TCA cycle into the biosynthesis of the polyketide precursor malonyl-CoA (de Carvalho Lima Lobato *et al.*, 2007). The putative AccA1/CpkK Acyl-CoA carboxylase complex (Pawlik *et al.*, 2007) is predicted to form malonyl-CoA from acetyl-CoA and

other acyl-CoA species (Rodriguez and Gramajo, 1999). In this case, homeostasis of the TCA cycle may be impaired and its metabolic balance then depends on 3-phosphoglycerate and anaplerotic reactions (de Carvalho Lima Lobato *et al.*, 2007), such as the one suggested for CpkG.

In total six malonyl-CoA units are used as starter and extender units in the formation of a first aliphatic, unsaturated C_{12} CPK polyketide intermediate by the Type I polyketide synthase (PKS) subunits CpkA, CpkB and CpkC (Pawlik *et al.*, 2007). The thioesterase ScoT most probably removes acyl residues different from malonyl-CoA that block the PKS extension modules in CpkABC (Kotowska *et al.*, 2002; Kotowska *et al.*, 2009). The primary polyketide may subsequently be further modified by CpkE, a predicted epoxide hydrolase, CpkI, a putative 3-oxoacyl-ACP reductase, CpkJ, a nucleoside-diphosphate-sugar epimerase homologue, the hypothetical protein CpkL, and by CpkD (Pawlik *et al.*, 2007). Although CpkD is predicted to be a secreted FAD-binding protein in view of a Twin-Arginine motif in its putative signal peptide (Signal P and (Pawlik *et al.*, 2007)), experimentally, it could not be verified as substrate for the Tat secretion system (Widdick *et al.*, 2006).

The tailored *cpk* precursor polyketide could then be transported out of the cell via the putative transmembrane efflux protein CpkF (Pawlik *et al.*, 2007). CpkH and ScF are Tat-dependent, cell wall-associated (Widdick *et al.*, 2006) FAD-binding oxidoreductase homologues (Pawlik *et al.*, 2007). Based on the phenotype of a *scF* mutant that produces abCPK, but not yCPK (chapter 3), we propose that the precursor is first converted into the antibiotic abCPK by CpkH, and then subsequently modified to the yellow pigment yCPK by ScF. In *S. coelicolor* liquid cultures, abCPK could be extracted best from the cells, in contrast to the secreted yCPK (chapters 2 and 3). This is in agreement with the localisation of CpkH and ScF in the cell wall: although also found diffusing into solid media (chapter 3), the intermediate abCPK may mainly stay attached to the cell before being further converted to yCPK.

ScF and CpkH show high sequence homology (blastp) and protein structure similarity (M. W. Fraaije, personal communication) to AknOx, a secreted oxidoreductase in *S. galilaeus* involved in aclacinomycin biosynthesis. AknOx catalyses the modification of the sugar residue of this antibiotic (Alexeev *et al.*,

2007). Also, pyridoxal phosphate (PLP) dependent transaminases, such as CpkG, are involved in the synthesis of aminosugars that subsequently serve as glycone moiety in *Streptomyces* secondary metabolites (Liu and Thorson, 1994) (e.g. DnmJ in daunosamine biosynthesis in the doxorubicin producer *S. peuceticus* (Hutchinson and Colombo, 1999)). Interestingly, with FdtB from *Aneurinibacillus thermoaerophilus*, among several amino group donors tested, including glutamine, alanine, and aspartate, only glutamate resulted in a turnover (Pfoestl *et al.*, 2003). This may suggest a putative role of glutamate and CpkG in aminosugar synthesis in the *cpk* biosynthetic pathway. This would be consistent with a function of CpkG in the direct transamination of a *cpk*-related metabolite proposed above. Furthermore, also the epimerase CpkJ is predicted to act on sugars (Pawlik *et al.*, 2007). A likely function of CpkJ is the conversion of a C-1 activated nucleoside diphospho-sugar, typically the TDP-D-glucose derivative TDP-4-keto-6-deoxy-D-glucose. Usually, the resulting activated deoxy-L-hexose isomer is then subsequently incorporated in glycosylated antibiotics (Walsh *et al.*, 2003). All these findings suggest the glycosylation of an intermediate of the *cpk* biosynthetic pathway and the presence of a sugar moiety attached to the polyketide aglycon of yCPK and abCPK.

On the other hand, the *cpk* gene cluster does not provide various additional gene functions needed for, e.g., glycon synthesis and also no glycosyltransferase is predicted in the *cpk* gene cluster (Pawlik *et al.*, 2007). Generally, the genes encoding the dedicated glycosyltransferases are found in the same gene cluster that contains the genes to make the particular activated deoxysugars and the aglycon (Walsh, 2002).

However, we cannot exclude that the missing enzymatic functions for the putative glycosylation of the *cpk* product are encoded elsewhere on the *S. coelicolor* chromosome, outside of the *cpk* cluster. In the elloramycin producer *Streptomyces olivaceus* it has been demonstrated that a small glycon biosynthesis gene cluster and the polyketide aglycon biosynthesis gene cluster are present on separated chromosomal loci (Ramos *et al.*, 2008). In *Saccharopolyspora spinosa*, only one set of biosynthetic genes is present providing rhamnose for both primary structural components (cell walls) and a secondary metabolite (spinosyns). Also in *S. spinosa* the sugar biosynthesis genes are found far away from the spinosyn aglycon gene

cluster (Madduri *et al.*, 2001). Biosynthesis of the heterocyst envelope polysaccharide (HEP) in the filamentous cyanobacterium *Anabaena sp.* is mainly encoded in the HEP genetic island. Glycosyltransferases essential for HEP production, however, are located outside the HEP island (Wang *et al.*, 2007). The given examples suggest that also in yCPK and abCPK production yet unidentified gene functions may be encoded outside of the *cpk* gene cluster, involved in synthesis and attachment of a putative glycon moiety. CpkG, CpkJ, and possibly also glutamate, may be involved in deoxy-sugar biosynthesis; CpkH and ScF may successively modify the glycon resulting in abCPK and yCPK, respectively. Structure elucidation of the *cpk* metabolites may eventually reveal whether they have one or more sugars attached, or whether a sugar-like structure is present in an unglycosylated *cpk* polyketide.

CpkN is a predicted *Streptomyces* antibiotic regulatory protein (SARP) and overexpression of CpkN leads to enhanced production of yCPK (Pawlik *et al*., poster 47, Biology of *Streptomyces*, Münster, Germany, 2009). Its function may therefore resemble that of CpkO, the other SARP regulator and pathway-specific activator of the gene cluster. A third, presumably negative, regulatory gene, ScbR2, together with CpkO may constitute negative feedback regulation of the *cpk* genes (chapter 2, Fig. 2). As yet, for none of these transcriptional regulators DNA binding sites and direct target genes have been characterised.

Via CpkO that is under the direct control of ScbR (Takano *et al.*, 2005a), regulation of the *cpk* gene cluster is linked to the *S. coelicolor* butanolide system (Fig. 2). The results presented in this thesis thus provide for the first time a continuous regulatory cascade from the bacterial hormones (the γ-butyrolactones) to secondary metabolites (abCPK and yCPK) in *S. coelicolor* (chapters 2 and 3). Furthermore, the identification of abCPK is the first successful example of the induced production of a hitherto undescribed antibiotic in a streptomycete by the manipulation of a regulatory gene (*scbR2*) (chapter 3). This concept could be applied not only to the *cpk* gene cluster, but to virtually all orphan gene clusters; not only in *S. coelicolor* but essentially in all streptomycetes and even beyond the genus. Hence, awakening these sleeping genes affords a promising chance for the discovery of countless novel secondary metabolites and therewith potentially new antibiotics.